

Regeneration and induced polyploidy in ferns

ELVA LAWTON

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REGENERATION AND INDUCED POLYPLOIDY IN FERNS¹

ELVA LAWTON

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INTRODUCTION

Induced apospory, or the regeneration of prothalli from the young leaves of ferns, has been known since 1908, when Goebel (8) described the appearance of prothallial tissue on the detached primary leaves of members of the Polypodiaceae and the Cyatheaceae. *Pteris longifolia* regenerated prothalli from the surface of the leaf and from the petiole. Rhizoids and sex organs were present on the prothalli, but sporophytes never appeared. *Ceratopteris thalictroides* produced prothallus-like structures which bore rhizoids and antheridia, and also stomata near the antheridia. Some leaves of this same fern developed buds on the petioles from which there arose structures intermediate between leaves and prothalli. *Gymnogramme chrysophylla* also produced prothallus-like outgrowths which contained stomata. When the primary leaves of *Polypodium aureum* were cut off, sporophytes grew from the buds produced on the epidermal cells. *Alsophila van Gertii* produced adventitious buds on the leaves, typical prothalli, and intermediate structures such as prothalli containing stomata. Later Goebel (9) described regeneration from a third family. Primary leaves of *Osmunda regalis* developed typical prothalli bearing rhizoids.

Woronin (25) made similar experiments with the leaves of some apogamous ferns as well as with some which reproduced sexually. *Trichomanes Kraussii* of the Hymenophyllaceae was apogamous and its leaves developed prothalli by apospory, especially if they were cut off and kept growing on a suitable substratum. The apospory could go still further in that the border cells of the leaves produced antheridia. This condition was termed "apoprothallia." Two species of *Pellaea*, both of which were apogamous, were capable of regenerating. *Pellaea nivea* produced filamentous prothallus-like outgrowths from the surface cells of detached leaves. Only the primary leaves of *Pellaea flavens* regenerated; but sometimes the secondary leaves

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which were cut off continued to grow until they were much larger, and the blade was very irregular in outline. The primary leaves often grew in the same manner and they produced other structures as well. Numerous rhizoids developed on the petioles of some of the leaves, while others produced adventitious buds. Two non-apogamous ferns reacted in much the same manner. Buds which gave rise to leaves and roots developed both on the blade and on the petiole of *Notochlaena Marantae*. Small prothalli bearing rhizoids and antheridia developed from the petiole of a detached leaf of *Gymnogramme farinifera*.

Brown (3) obtained regeneration from the petiole of a leaf of *Phegopteris polypodioides*. A cellular mass resembling a prothallus was formed and from it rhizoids developed, as well as true leaves and structures intermediate between prothalli and true leaves.

Heilbronn (10) described the formation of rhizoids and sporophytic buds on detached leaves of *Cystopteris fragilis*.

Many of the regenerating leaves described by Goebel and Woronin developed structures composed of both prothallus-like cells and cells which were typically sporophytic, such as tracheids and stomata. Lang (12) has described some extreme instances of the production of sporophytic and gametophytic structures without definite sequence. The prothalli of Lang's cultures were grown from spores. *Scolopendrium vulgare* var. *ramulosissimum* and *Nephrodium dilatatum* var. *cristatum gracile* produced sporangia on the prothalli, sometimes apparently from transformed archegonia. Archegonia were present on the same prothalli with sporangia; and reproduction was both apogamous and sexual. Lang (14) reported a similar situation in *Scolopendrium vulgare* var. *ramo-digitatum*. Some of the sporangia produced spores which seemed to be mature, but they did not grow.

Pace (19) reported the presence of sporangia on prothalli of a fern which was probably one of the Osmundaceae.

Since the literature relating to apospory and apogamy in general has been reviewed by a number of other writers it is not discussed here (Allen, 1; Ernst, 5; Farmer and Digby, 7; Steil, 23).

In all the earlier works on induced apospory in ferns the regenerated prothalli did not develop beyond the production of sex organs. In 1925 Heilbronn (11) reported a study of *Polypodium aureum*, the primary and secondary leaves of which regenerated to form adventitious buds, filamentous prothalli, and typical heart-shaped prothalli. Tetraploid sporophytes developed from the regenerated prothalli which differed from the diploid plants not only in chromosome number but also in cell size.

In addition to the studies on regeneration in the ferns, the Bryophytes have been the subject of many similar experiments. Gametophytes were produced from sterile cells of capsules of *Anthoceros* by Lang (13) and by Schwarzenbach (21), but sporophytes were not obtained from these re-

generated structures. Éliè and Émile Marchal (16-18), Wettstein (24), and LaRue (15) have induced apospory in various species of mosses. The Marchals produced tetraploid sporophytes bearing diploid spores. In some species tetraploid gametophytes were obtained by regeneration from tetraploid capsules. By regeneration Wettstein obtained bivalent, trivalent, and quadrivalent races from capsules having the $2x$, $3x$, and $4x$ chromosome numbers. Octoploid gametophytes were produced from capsules of intergeneric hybrids.

Polyploidy in ferns has not been as extensively investigated as has polyploidy in the mosses. With the exception of the tetraploids developed by Heilbronn (11) in a single species, no fern sporophytes with more than two complete sets of chromosomes have been produced experimentally. The apparent neglect of studies on the ferns has probably been due rather to the difficulty of the material than to any lack of interest.

It seemed desirable to extend the work along different lines and this paper deals with attempts to solve a number of problems, some of which have been suggested by similar investigations in the mosses. Attempts were made to induce apospory and the production of tetraploid sporophytes in as many species as possible, and to investigate the conditions under which regeneration occurred. Since the tetraploid capsules of mosses regenerated gametophytes, it was believed that prothalli with the $4x$ chromosome number could be produced from leaves of tetraploid sporophytes of ferns. The present work deals, also, with attempts to produce triploid sporophytes by crossing $2x$ gametes and x gametes. It was desirable to show that fertilization was necessary for the production of sporophytes on regenerated gametophytes and to demonstrate fertilization in the $2x$ prothalli. Apospory and apogamy as they occur in nature are closely related to events resulting from the cultivation of detached leaves of young sporophyte plants; and the further study of induced apospory may throw some light on abnormalities in life cycles. Further evidence that apogamy does not necessarily follow induced apospory was needed.

METHODS

Cultural Procedure

Since all prothalli and young sporophytes were obtained by growing them under controlled conditions, the success of the entire investigation depended on the cultural methods and the technique of handling the material. Therefore the development of the method will be described in greater detail than would otherwise be necessary.

Leaves or parts of leaves with ripe sporangia were thoroughly washed under running water in order to remove foreign fern spores and mold spores; then washed several times in sterile distilled water and put in sterile Petri dishes to discharge their spores. When they were planted they were

transferred with sterile instruments. The medium which was found to be most satisfactory for nearly all of the ferns grown was .12 percent Shive's nutrient solution. All solutions were sterilized in the autoclave.

The spores were planted in small glass capsules on the solution and the cultures were kept in a greenhouse where they never received direct sunlight. The spores germinated to form prothalli floating on the surface; and the young prothalli were transferred to fresh solution every three or four weeks and thinned out in order to give them plenty of room to develop. In this way they grew to maturity and formed sex organs and young sporophytes. Usually some of the sex organs were immersed in the solution so that fertilization took place; but if the sporophytes were slow to form, especially in ferns where the antheridia are not produced in such large numbers, shaking the dish brought about the opening of the sex organs and fertilization.

The time required for germination varied from two days to four weeks according to the species and the age of the spores. Young sporophytes with one or two leaves ready for regeneration experiments were formed from two to five months after the spores were planted, the time varying with different ferns.

If the prothalli showed a tendency to sink, especially after they had been disturbed in order to bring about fertilization, they grew better if transferred to capsules containing three quarters of an inch of sterilized white sand soaked with the nutrient solution.

The common contaminations were algae and fungi, although washing the spore-bearing leaves usually removed most of the mold spores. Numerous collections were made at different times and from different parts of the plant; and each collection was preserved in separate, sterile Petri dishes. The collections which were found to be contaminated could be discarded after the first trial plantings. In the early stages of the work methods of control were tried which would rid the cultures of contaminations. A very weak solution of copper sulfate would sometimes kill algae without injuring the prothalli; and a weak solution of potassium permanganate would often free the cultures from fungi. However, a better method was to grow large numbers of capsules of the prothalli in order that any which became contaminated could be discarded. All cultures were kept in a clean place in the greenhouse. When a dish was to be examined, the outside was wiped off with alcohol before it was opened.

The above culture method was used for all the ferns except *Scolopendrium* and *Pteris*. Knopf's nutrient solution was found to be better for *Scolopendrium*. *Pteris* was grown on sterilized sphagnum soaked with Shive's nutrient solution. Agar made with a .24 percent Shive's solution was used in experiments to prevent fertilization. Prothalli with immature archegonia were transferred from solution to Petri dishes containing agar. The prothalli grew to be very large and produced numerous archegonia if they were transferred to fresh agar every two or three weeks.

Contaminations were more common on agar than on solution, and they often appeared soon after the prothalli were transferred to fresh agar. Frequent sterilization of the needles in a flame during the process of removing the prothalli to a dish of fresh agar was necessary. When a dish containing prothalli to be transferred was brought from the greenhouse, the surface was washed with alcohol to remove sources of contamination.

In all the species regeneration was brought about by cutting the young leaves off with sterile needles, and transferring them to fresh .12 percent Shive's solution. The leaves usually floated on the surface; but if there was a tendency to sink, they were supported by sand. From ten to twenty-five leaves were placed in each capsule containing approximately 30 cc. of the sterile solution. The first, second, and third leaves were cut off as they were formed on the embryo; but regeneration was not very common in the third leaves. Wounding seemed, in some cases, to induce regeneration since the prothalli often developed where the leaf had been injured in cutting. Regeneration usually took place within two to four weeks after the leaves were cut off, but in a few species the leaves remained green for three months before regeneration started.

It was necessary to exercise great care in the preparation of leaves for regeneration in order to prevent contaminations. All apparatus and solutions were sterilized, and the leaves were cut and transferred as quickly as possible. Good results were never obtained in any culture contaminated with green algae or blue-green algae. The nutrient solution was a very favorable place for algae, which grew much faster than the tissue regenerated from the leaves. Since the regenerated prothalli formed in contaminated cultures were always crowded out by the algae and died in an early stage, it was important to prevent contaminations and to remove all contaminated capsules from the greenhouse immediately. Mold contaminations were not so harmful since conditions were not favorable for the growth of fungi, and they usually died in a few weeks. All the leaves which died without regenerating were removed because fungi could grow on them. It was often necessary to transfer all the green leaves to clean, sterile dishes of solution when some of the leaves died and became moldy.

In a few cultures where algae appeared after the regenerated prothalli were well established, the $2x$ prothalli were saved by transferring them to soil. The soil was not sterilized, since the prothalli grew better on unsterilized soil. Contaminations by fern spores were controlled by keeping the pots of soil moist and under glass covers for several weeks. Under such conditions, any foreign fern spores would germinate and could be removed before the $2x$ prothalli were put on the soil. All prothalli grown on soil were kept under glass covers, and were either watered from below or with sterile water.

Technique

The cell measurements were made with an ocular micrometer on material mounted in lactic acid according to the method described by Simpson (29). The leaves or prothalli were kept in lactic acid in a warm place until they became clear and then mounted. The areas of the epidermal cells were obtained by measuring camera lucida drawings of the cells with a planimeter. The actual area of the cell was determined by dividing the area derived from the planimeter measurement by the magnification of the drawing. The areas of the prothallial cells were obtained by multiplying the lengths by the widths since the cells were approximately rectangular.

The sporogenous tissue used for chromosome counts in *Aspidium marginale* was fixed in Flemming's medium solution. All other material for chromosome counts was fixed in formol-acetic-alcohol with the following formula: 50 percent alcohol, 100 cc.; formalin (neutralized by magnesium carbonate), 7 cc.; glacial acetic acid, 7 cc.

The sections were cut six microns in thickness and stained with Flemming's triple stain or with gentian violet alone according to Newton's method.

EXPERIMENTS

Regeneration was tried in sixteen different species of ferns from the Polypodiaceae and the Osmundaceae, but regenerated structures were never obtained from five of this number, as follows; *Osmunda Claytoniana* L., *Onoclea sensibilis* L., *Camptosorus rhizophyllus* (L.) Link., *Aspidium spinulosum* var. *intermedium* (Muhl.) D.C. Eaton, and *Pteris aquilina* L. Since the numbers of leaves of the five ferns used in these cultures were small, it is impossible to draw any conclusions as to their ability to produce prothalli by induced apospory.

Several other ferns which did not prove suitable for an extensive study formed prothalli by induced apospory. *Scolopendrium vulgare* Sm. and *Dicksonia punctilobula* (Michx.) Gray formed outgrowths from the tips of the lobes of the primary leaves. It was difficult to determine where the sporophytic tissue of the leaf ended and the gametophytic tissue of the prothallus began in some of these structures. Sometimes filamentous prothalli similar to those described for *Woodsia obtusa* were present. No sex organs were formed and none of the regenerated tissue lived longer than a few weeks.

Polystichum acrostichoides (Michx.) Schott regenerated heart-shaped prothalli which bore antheridia. These prothalli grew for more than a year and became very large but had never developed archegonia when they became contaminated with green algae and died. The prothalli grown from spores produced few archegonia in these cultures, even when they had grown for a long time and were very large.

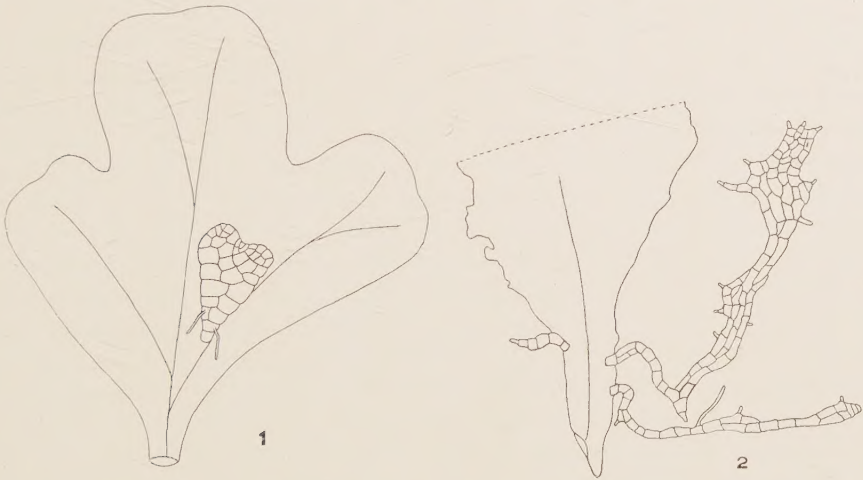
Asplenium platyneuron (L.) Oakes regenerated from primary leaves, secondary leaves, and roots. Both the leaves and the roots developed

filamentous prothalli, heart-shaped prothalli, and sporophytic buds. Antheridia were borne on the heart-shaped prothalli but no archegonia were present when this culture became contaminated with green algae and died.

Cystopteris fragilis (L.) Bernh., *Osmunda regalis* L., and *Athyrium filix-femina* var. have regenerated heart-shaped prothalli with archegonia and antheridia from which sporophytes developed. A report of the studies on these three ferns will be published later.

***Pteris cretica* var. *albo-lineata* Hook.**

The spores of this fern were obtained from the commercial florist J. F. Anderson, Short Hills, New Jersey. They were planted in glass capsules on a medium of sterilized sphagnum soaked with Shive's nutrient solution. The gametophytes developed very quickly, forming heart-shaped prothalli, but never bearing archegonia. Six weeks after the spores were planted, nearly all the prothalli formed apogamous sporophytes in the manner described by Farlow (6) and deBary (2) for *Pteris cretica*. While the sporophytes were quite young (having only two or three leaves) and while they were still growing on the sphagnum, the primary leaves of about thirty were cut off and put on Shive's solution. Five weeks after the



TEXT FIG. 1. *Pteris cretica* var. *albo-lineata*. Primary leaf regenerating a heart shaped prothallus.

TEXT FIG. 2. *Woodsia obtusa*. Leaf with three regenerated filamentous prothalli.

leaves were cut off, regeneration had begun on the dorsal surface of one leaf near a vein. Very soon the regenerated tissue formed rhizoids and broadened out to form a heart-shaped prothallus (text fig. 1). The development of this prothallus resembled the development of the prothalli of *Pteris cretica* var. *albo-lineata* grown from spores. No archegonia were formed on the regenerated prothallus, and no antheridia were observed.

About eight weeks after this prothallus was regenerated, it produced an apogamous sporophyte in the same manner that the original apogamous sporophytes in this species were developed. This apogamous sporophyte grew for several months and produced a number of leaves and roots. The regeneration described above was the only case in *Pteris cretica* var. *albo-lineata*.

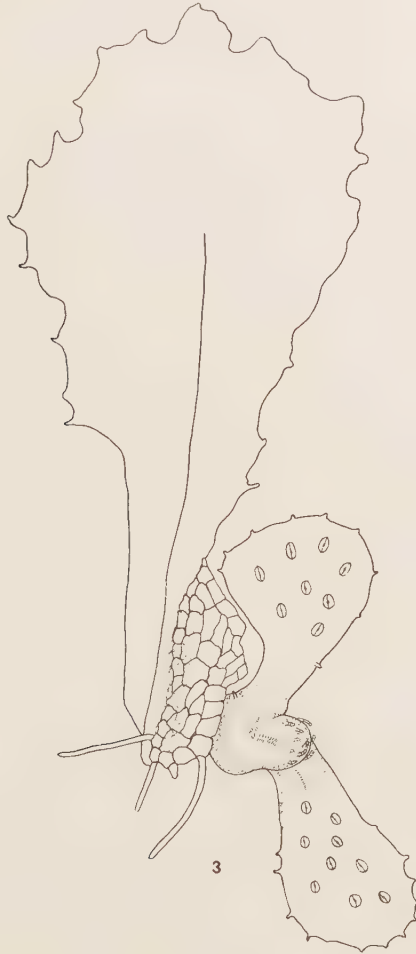
***Woodsia obtusa* (Spreng.) Torr.**

Spores of *Woodsia obtusa* were collected from a plant growing in cultivation near Cold Spring Harbor, Long Island. They were planted on Shive's solution, and the young sporophytes developed on the gametophytes floating on the solution. Approximately 1500 leaves, 75 percent of which were primary leaves, were cut off to induce regeneration; 6.25 percent of the leaves regenerated but no normal heart-shaped prothalli were ever formed. No archegonia developed on any of the regenerated tissue, and probably no antheridia, since none were ever found although a large number of the prothalli were examined carefully. There were two different types of regeneration: filamentous prothallus-like structures, and sporophytes with true leaves and roots. The two kinds of structures were often found on the same leaf and they developed either from the petiole or from the blade.

The filamentous structures consisted of undifferentiated cells similar to normal prothallial cells and bore rhizoids (text fig. 2). They never became very large and usually lived only a few weeks. Text figure 3 shows a young sporophyte developing at the base of the petiole of a leaf which had been cut off and cultivated on solution for more than two months. The shape of the leaf was modified by growth in the marginal region, giving it a very irregular outline. Growth of this type occurred more often in leaves which were cut off before they had reached full size. On the leaf shown in text figure 3, prothallial tissue was formed before the regenerated sporophyte developed. Two leaves and a growing point grew from the mass of cells where the prothallus was attached to the petiole, the growing point later giving rise to leaves and roots. This illustration shows the development of a prothallus followed by the development of true leaves. Other regenerating leaves developed prothallial tissue first, then a series of structures intermediate between prothalli and leaves, and finally true leaves.

Regeneration in *Woodsia* took place in the roots also. Plants grown in glass capsules on a clear solution develop chlorophyll in the roots, especially in the meristematic tissue at the tip. When the green roots were cut off and kept in nutrient solution for two or three months, they began to regenerate. The tissue of the root tip within the root cap became greener and grew, forming a spherical mass which broke through the root cap. This mass of cells developed further in much the same manner as the stem initial in a normal embryo, and formed leaves and roots. The young sporophyte remained attached to the root which regenerated it. It resembled a sporophyte developed from a fertilized egg; and when it was

transferred to soil, it grew to maturity. Nearly all the regenerating roots formed sporophytes in the manner described; but a few formed filamentous prothalli which were similar to those formed on the regenerating leaves. They developed from the root tip or from the cells a short distance from the tip. The writer is not aware that regeneration of prothalli from roots has been described previously, although the formation of sporophytes on uninjured roots or on detached roots of *Ophioglossum* has been described



TEXT FIG. 3. *Woodsia obtusa*. Sporophytic bud formed on prothallial tissue regenerated from the petiole of a leaf. Leaf showed growth in the marginal region.

by Campbell (4) and by Poirault (20). The roots of *Woodsia* regenerated the same types of structures as the leaves of that species.

Shive's and Knopf's solutions were both used in the regeneration of *Woodsia*. Two series of solutions of different concentrations varying from

.3 grams per liter to 5 grams per liter were tried in order to test the effect of the medium. About 200 detached leaves were cultivated on the different concentrations of each solution. The two solutions gave approximately the same results in regard to the number of leaves which regenerated and the structures produced; but contaminations by algae were more common in Knopf's solution. Both filamentous prothalli and sporophytic buds were formed in all the concentrations of both solutions. The number of sporophytes was slightly larger in the higher concentrations of both solutions; and in general, more leaves regenerated in the solutions of medium concentrations. Heart-shaped prothalli were never formed and all the regenerated tissue either died or formed sporophytes capable of developing to maturity.

***Aspidium marginale* (L.) Sw. (Long Island)**

The spores of *Aspidium marginale* were collected at Cold Spring Harbor, Long Island, New York. Regeneration in this species took place readily, usually about four weeks after the leaves were cut off, and 33 percent of the 135 leaves which were cut regenerated. Growth began in the superficial cells of the blade either on the surface or on the margin as shown in text figure 4. In *Aspidium* it was noticed that regeneration often took place near an injured spot on the leaf. Only one case of the formation of a sporophytic bud as described for *Woodsia* was found and in this instance the regeneration was from the petiole.

The Regenerated Gametophytes

The first structures formed were filamentous, usually only one or two cells wide, and they bore rhizoids and a few antheridia. A growing point was very soon established in each and a true heart-shaped prothallus developed which grew to sexual maturity very quickly. The development after the short filamentous stage was not observed to differ from the normal development of a prothallus from a spore. The regenerated prothalli resembled the normal in that they were typically heart-shaped with the growing point in the notch and in that they produced archegonia on the thickened cushion and antheridia and rhizoids on the posterior part; but when cell measurements were taken it was found that the cells were larger. With the exception of the 4x prothallus, which is discussed below, the measurements were made on cells in the wings of sexually mature prothalli all of which had been grown under like environmental conditions. Gametophytes vary under different external conditions, such as differences in substratum and in the amount of light. Also under crowded conditions normal development is prevented; the prothalli are smaller and the cells themselves are smaller. Therefore the *x* and the *2x* prothalli were grown on the same solution and under the same conditions as to light and temperature; and those on which the cell measurements were taken were chosen from cultures having approximately the same number of plants to a given

amount of nutrient solution. Normal and regenerated prothalli were selected which were nearly the same size, and which bore sporophytes at the same stage of development. The length and the width of each of four hundred cells was measured from both the normal and the regenerated prothalli and the variates were taken from a number of different plants. The results of the measurements of the prothallial cells are shown in table 1.



TEXT FIG. 4. *Aspidium marginale*. Heart shaped prothallus regenerated from the margin of a leaf. Filamentous prothalli were formed first.

The length of the $2x$ cells did not show much increase but it was significant since the difference in lengths was six times the probable error. The width showed a much greater increase and the difference in widths was twenty-seven times the probable error.

Chromosome counts were made from cells of the developing antheridia

TABLE I. *Cells of Prothalli of Aspidium marginale (Long Island)*

Prothallus	No. Cells Measured	Lengths in μ	Difference from (a)	% Increase over (a)
(a) x	400	117.19 $\pm$.87		
(b) $2x$	400	125.47 $\pm$ 1.06	8.28 $\pm$ 1.37	7
(c) $4x$	100	112.53 $\pm$ 1.61	4.66 $\pm$ 1.83	
Widths in μ				
(a) x	400	49.97 $\pm$.24		
(b) $2x$	400	62.67 $\pm$.39	12.70 $\pm$.46	25.4
(c) $4x$	100	90.32 $\pm$ 1.16	40.35 $\pm$ 1.18	80.0
Areas in sq. μ				
(a) x		5900		
(b) $2x$		7900		34
(c) $4x$		10200		73

in both the normal and regenerated prothalli. The number in the prothalli grown from spores was $36 \pm$, while $65 \pm$ chromosomes were found in the regenerated prothalli. The dividing cells were rare, and it was necessary to section many prothalli bearing young antheridia in order to find a sufficient number of cells in the proper stages to make chromosome counts. A number of counts were made; but, due to the large number of chromosomes and the fact that they are long and slender, it is almost impossible to be sure of the exact number from counts made in somatic tissue. It seems certain from the above numbers that the diploid number is carried over into the regenerated prothalli.

Observations were made on mature sex organs and by means of paraffin sections on various stages in their development. Some antheridia on the regenerated gametophytes produced 32 sperms while others bore 64; the same numbers were observed in various antheridia on the normal prothalli. The antheridia of the $2x$ prothalli were seen to open; the sperms were motile and were attracted to the archegonia; sperms were found in the necks of the archegonia sectioned in paraffin. The archegonia did not differ from the normal ones except perhaps by a slight increase in size. Unopened archegonia were found showing the egg, the ventral canal cell, and the neck canal cell with its two nuclei.

Although fertilization has not been found in this species, there is reason to believe that it took place in a normal fashion. Fertilization was found in the $2x$ prothalli of another fern which is described below; and it is to be expected that it will be demonstrated in this species also.

The Sporophytes Produced by the Diploid Gametophytes

Sporophytes were formed about four months after the regenerated prothalli developed; and from the first it seemed probable that they were the result of fertilization, because they developed a few weeks after the prothalli had been shaken up in the solution on which they were growing. Since the $2x$ prothalli were grown on sterile media, and in dishes with lids,

there was no possibility of contamination by other ferns. The sporophytes developed from archegonia because the young embryos were observed in the enlarged venters with the brown necks of the archegonia still attached; and the sections showed a well developed foot. No record has been found in the literature that apogamy or parthenogenesis occurs in the normal *Aspidium marginale*. Paraffin sections of $2x$ prothalli bearing archegonia and young embryos showed that the course of development was normal. Two celled embryos showed that the first cell wall was parallel to the axis of the neck of the archegonium as in the normal embryos. Four, eight, and sixteen celled embryos were found as well as later stages.

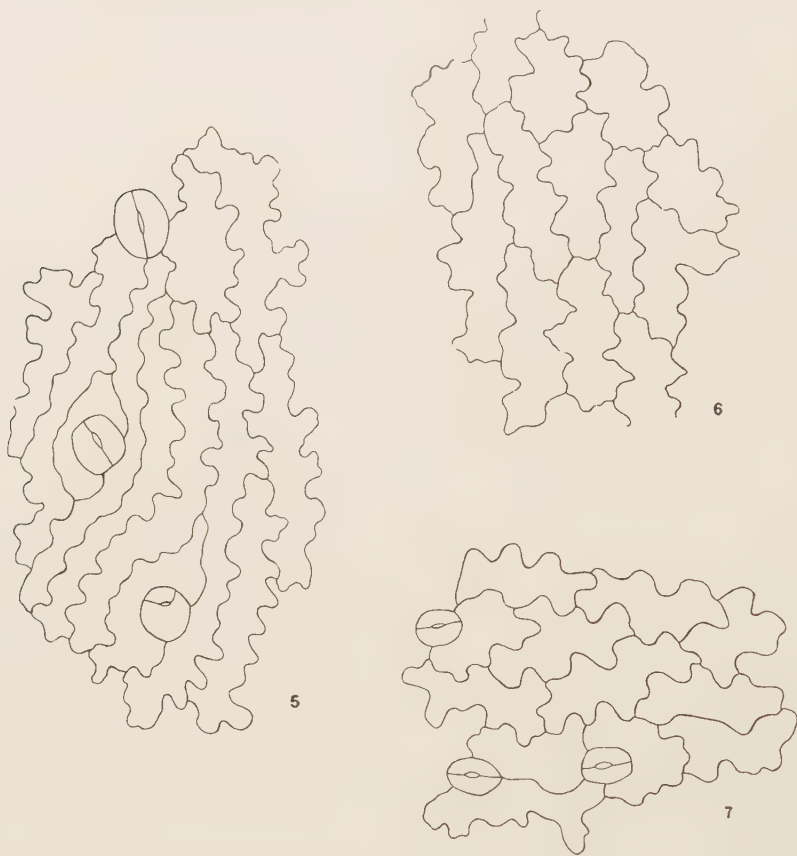
Due to the rapid growth of prothalli and their tendency to regenerate smaller prothalli, a large number of $2x$ gametophytes was available for an experiment on fertilization. The prothalli grown on sand or on solution never became very large without producing embryos, there being opportunities for fertilization to take place every two or three weeks when they were transferred to fresh solution, and the sex organs in contact with the solution opened. Therefore a number of small prothalli either with no archegonia or with very small ones were removed to agar. In a few weeks numerous mature archegonia were present on all the prothalli on agar; and they continued to form in large numbers, as the prothalli grew to be several times as large as those growing on sand or on solution. About 150 prothalli were kept on agar for periods varying from one to four months. Only ten sporophytes appeared on these gametophytes and they probably developed because a few drops of water condensed on the lids of the Petri dishes and dropped on the prothalli. The small number of sporophytes formed on agar, contrasted with the fact that all prothalli on sand or solution produced embryos, made it seem highly probable that all the sporophytes were the result of fertilization.

In order to obtain more direct evidence that fertilization was necessary for the formation of sporophytes, a dish containing about fifty prothalli with mature sex organs was filled with nutrient solution for twenty-four hours. Other dishes of similar prothalli with mature sex organs were not filled with solution. After five weeks forty-three young sporophytes were counted in the dish which had been filled with solution. All the sporophytes were approximately of the same size, showing that the archegonia had all been fertilized at the same time. The other dishes with prothalli having mature sex organs which had not been in solution did not develop sporophytes.

The evidence from the chromosome numbers strengthens the theory that fertilization took place. Chromosome counts were made in the cells of the first leaf of the embryo where mitotic figures were abundant. The very large number of chromosomes made it impossible to be sure of the exact number, although numerous careful counts were made on a large number of cells. It seemed certain that at least 120 chromosomes were

present, since the counts varied from 120 to 130 in different cells. When this number was compared with the numbers found in the gametophytes there was every reason to believe that the sporophytes formed on the regenerated prothalli were tetraploids.

A comparison of the young diploid and tetraploid sporophytes showed some differences. The leaves of the tetraploid were more irregular in outline, in some cases showing peculiar thickenings and foldings on the



TEXT FIG. 5. *Aspidium marginale*. Lower epidermal cells of tetraploid sporophyte.

TEXT FIG. 6. *Aspidium marginale*. Upper epidermal cells of tetraploid sporophyte.

TEXT FIG. 7. *Aspidium marginale*. Lower epidermal cells of diploid sporophyte.

surface. A microscopic study of the fully developed primary leaves of diploid and tetraploid plants at the same stage of development showed variations in size of stomata, and variations both in the size and shape of the epidermal cells (text figs. 5, 6, 7, and 8). Table 2 shows the numbers of measurements made and the sizes of the cells, in microns, of the diploid

and tetraploid plants. A number of plants were used for all the measurements of the different kinds of cells.

TABLE 2. Cells of Leaves of *Aspidium marginale* (Long Island)

Sporophyte	No. Cells Measured	Stomata Lengths in μ	Difference	% Increase
2x	300	42.77 $\pm$.19		
4x	300	54.11 $\pm$.25	11.34 $\pm$.32	26
		Upper Epidermis Lengths in μ		
2x	200	101.59 $\pm$ 1.07		
4x	200	129.14 $\pm$ 1.26	27.55 $\pm$ 1.65	27
		Lower Epidermis Lengths in μ		
2x	350	119.45 $\pm$ 1.11		
4x	350	233.22 $\pm$ 2.18	113.77 $\pm$ 2.44	95
		Lower Epidermis Widths in μ		
2x	300	60.61 $\pm$.49		
4x	300	63.56 $\pm$.61	2.95 $\pm$.78	4.8
		Areas in sq. μ		
		Lower Epidermis		
2x		3952		
4x		6916		75

The lengths of two hundred lower epidermal cells of full grown leaves of a mature plant were measured. The mean was 119.62 $\pm$.92 microns. When this was compared with the length of the corresponding cells of normal primary leaves it was found that the difference was less than the probable error. Therefore it is to be expected that the cells in the mature tetraploid sporophytes will be approximately of the same size as the cells of the primary leaves of tetraploid sporophytes.

The difference in widths of the lower epidermal cells of the 4x and 2x plants is only 3.8 times its probable error. In all other measurements the differences are so great that there can be no question of their significance. By means of a planimeter the areas of twelve cells of the lower epidermis of the diploid plant, and the areas of the same number of cells of the tetraploid plant, were measured from camera lucida drawings. Cells were selected whose measurements corresponded on the average to the dimensions given in table 2. From the figures thus obtained the areas of single cells in square microns were computed.

In his description of the tetraploid plants of *Polypodium aureum*, Heilbronn (11) states that the areas of normal and tetraploid epidermal cells are to each other as 1 : 2.2. Therefore the epidermal cells of tetraploid plants of *Aspidium marginale* do not show as great an increase in size as the epidermal cells of *Polypodium aureum*.

Regeneration from Tetraploid Sporophytes

Regeneration was also obtained from leaves of the $4x$ sporophytes formed on the regenerated prothalli described above, giving prothalli with the $4x$ chromosome number. The $4x$ regeneration resembled the early stages of the $2x$, the gametophytes being filamentous and without a growing point, and sometimes developing fifty or sixty cells in a single row. One culture of $4x$ tissue obtained from a single leaf produced twelve or more prothalli at the end of nine months. Very few of these prothalli were truly



TEXT FIG. 8. *Aspidium marginale*. Upper epidermal cells of diploid sporophyte.

TEXT FIG. 9. *Aspidium marginale*. Prothallus regenerated from leaf of tetraploid sporophyte.

heart shaped although sex organs of both kinds were produced regardless of the shape of the prothallus. Structures similar to that shown in text figure 9 were common, having many segments which often ended in filaments of a single row of cells. The region marked "x" consisted of cells which had not reached full size and the notches in this lobe resembled growing points. A number of archegonia developed on a broad, irregularly ribbon-shaped prothallus which had developed a thickened region similar to a cushion along one edge, while the other side consisted of a single layer of cells like the wings of a normal prothallus.

One hundred measurements of the length and width of cells of the prothallus shown in figure 9 were made. The mean length was 112.53 ± 1.61 microns and the width was 90.32 ± 1.16 . Since the measurements were taken from a single prothallus, due to lack of material, and since that prothallus had not developed sex organs and was probably not at exactly the same stage of development as the other prothalli whose measurements are given in table 1, a comparison of the results obtained in the two instances may be misleading. However, it seems to be evident that the width of the $4x$ prothallial cells is greater than that of the $2x$ cells. A similar difference was observed in the comparison of the $2x$ prothallial cells with the ones having the x chromosome number.

The sex organs on the $4x$ gametophytes seemed to be normal but a more careful examination must be made when material is available. Although the same $4x$ tissue has been kept in culture for nearly a year, very few prothalli have developed because growth is very slow. It is hoped that prolonged culture will show that $4x$ prothalli can produce sporophytes with the $8x$ chromosome number.

***Aspidium marginale* (L.) Sw. (Pennsylvania)**

Spores of *Aspidium marginale* were also collected from a plant which had been brought from Penowa, Pennsylvania, and was growing in the Botanical Gardens at the University of Michigan. They were grown in order to repeat the experiments made with the same species collected on Long Island.

Chromosome counts made at sporogenesis showed that the haploid number was either 38 or 39. The similarity in the results of the experiments on the cultures from the two ferns suggests that they may be similar in constitution and that the chromosome numbers in the plant from Long Island may be

$$x = 38, \quad 2x = 76, \quad 4x = 152,$$

instead of

$$x = 36 \pm, \quad 2x = 65 \pm, \quad 4x = 120-130,$$

as obtained from the counts made in the vegetative divisions of the Long Island plant.

Approximately 800 leaves were detached from the young embryos of *Aspidium marginale* (Pennsylvania) and placed on solution in capsules; and as a result 25 percent of them regenerated. The early stages were filamentous and similar to the regeneration from the *Aspidium marginale* from Long Island; but in this experiment the heart-shaped prothalli were slow to develop and a large number of the filamentous structures died without producing them at all. This difference in regeneration in the two cultures may be due to some difference in constitution or to differences in external conditions resulting from the different seasons. The results of experiments made to determine whether regeneration in *Aspidium marginale* varied with the seasons were inconclusive.

The production of sporophytic buds on leaves cut off to induce regeneration was as rare in this culture as in the preceding one.

No chromosome counts were made in the regenerated prothalli, but there was no indication that they differed from the $2x$ prothalli of *Aspidium marginale* in which chromosome counts were made, and in all probability the chromosome number here is the $2x$ number also. The prothalli were similar to the ones in which counts were made and bore normal sex organs which produced sporophytes. From the studies on cell size described above, it is evident that the increase in length of the cells of the lower

epidermis of the leaf is the most marked characteristic of a young tetraploid plant of this species. Consequently, a study was made of the length of the lower epidermal cells in both the tetraploid and diploid plants of this culture, the stage of development of the embryo as a whole being the same as in similar studies made in the preceding culture.

When these measurements were compared with those made on *Aspidium marginale* from Long Island, it was found that the differences were not significant, being not more than twice their probable errors.

TABLE 3. *Cells of Leaves of Aspidium marginale (Pennsylvania)*

Sporophyte	No. Cells Measured	Lower Epidermis Lengths in μ	Difference	% Increase
2x	200	119.90 $\pm$ 1.62		
4x	200	225.11 $\pm$ 3.02	105.21 $\pm$ 3.42	88

Sporophytic Structures on Prothalli

A few abnormal plants developed, showing combinations of gametophytic and sporophytic characteristics. Text figure 11 shows a regenerated gametophyte which was heart-shaped but without a cushion and it consisted of a single layer of cells except for the mass of tissue which bore sporangia. Glandular hairs, such as are found on young leaves of sporophytes, were present on the tissue bearing the sporangia. Sections of the thickened region showed that it contained a few tracheids. The sporangia themselves (text fig. 12) were borne on stalks which were shorter and thicker than the typical stalks of the Polypodiaceae. Development ceased and the sporangia became a lighter green without forming mature spores and the annulus was never conspicuously differentiated. Sections showed a sporangial wall of a single layer of cells and the remains of what may have been a tapetum. The sporogenous tissue was in the sixteen cell stage in all the larger sporangia and the nuclei were in a resting condition. Since in no case more than sixteen cells were found, it appeared that the sporangia developed much like normal sporangia up to meiosis but at that point the nuclei became somewhat abnormal in appearance, and none of the sporangia showed any signs of the reduction division.

Another 2x prothallus developed a cushion and bore sporangia on one side of the prothallus and archegonia on the other side. A sporophyte developed from one of the archegonia.

Text figure 10 illustrates a plant whose posterior part was distinctly prothallial in character and consisted of a single layer of cells. The anterior part (in front of the dotted lines) resembled a leaf, having a mid-rib with tracheids, upper and lower epidermis, and a well developed mesophyll extending about half way from the mid-rib to the margin of the leaf. The mean length of these epidermal cells was found to be 113.70 $\pm$ 2.20 microns. The difference between the length determined for normal sporophytes and

this figure is only 2.33 times its probable error. Therefore these cells resemble the corresponding cells of the normal sporophytes, which might be expected since the chromosome number is in all probability $2x$ in each case. The mass of cells at the place where the gametophyte tissue meets



TEXT FIG. 10. *Aspidium marginale*. Plant regenerated from leaf of diploid sporophyte. The posterior part was prothallus-like and the part in front of the dotted lines was leaf like. It contained a mid-rib, epidermal cells, and stomata.

TEXT FIG. 11. *Aspidium marginale*. Prothallus regenerated from leaf of diploid sporophyte. Sporangia were borne on the thickened region.

TEXT FIG. 12. *Aspidium marginale*. Single sporangium from prothallus shown in figure 11.

the sporophyte tissue contained tracheids and when it first appeared seemed to be an early stage in the development of an apogamous sporophyte, but it never formed a leaf. Rhizoids were present both on this thickened structure and on the prothallial tissue.

Summary of Results of Experiments with Aspidium marginale

Regeneration from leaves of normal sporophytes gave gametophytes from which sporophytes were derived sexually. Chromosome counts were made in the normal and regenerated gametophytes, in the normal sporophytes, and in those produced on regenerated gametophytes. The chromosome numbers were doubled, giving $2x$ gametophytes and tetraploid sporophytes. The leaves of the $4x$ sporophytes regenerated prothalli bearing both kinds of sex organs. Probably the chromosome number in these gametophytes was the $4x$ number; but counts have not been made.

The cells of the regenerated prothalli, both the $2x$ and the $4x$, were increased in size, particularly in width. The cells of the $4x$ sporophytes were larger than those of the $2x$. Due to the great increase in length, but not in width, of the lower epidermal cells of the leaf, those cells were of a different shape from the corresponding cells of the $2x$ plant. A few regenerated prothalli were found bearing sporangia.

Woodwardia virginica (L.) Sm.

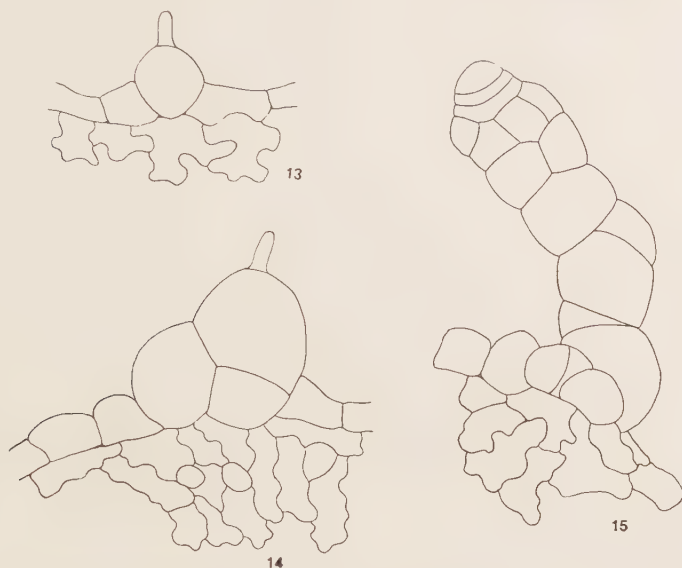
Spores of *Woodwardia virginica* were collected near Lake Ronkonkoma, Long Island, New York, and were germinated on Shive's nutrient solution. Regenerated prothalli appeared in about four weeks after the leaves were cut off and cultured. It was evident at once that it was particularly easy to induce regeneration in this fern, since 60 percent of the 50 leaves cut regenerated. Although the original plants were all grown on Shive's solution, both Shive's and Knopf's solutions were used for regeneration, and they seemed to be equally good.

The Plants Obtained by Regeneration

The regenerated prothalli developed from single cells, either on the margins or on the surfaces of the leaves. Text figures 13-15 show the development of a filamentous prothallus from a single cell. A mass of filamentous prothalli covered with antheridia developed on the regenerating leaves first, and from the filamentous gametophytes heart-shaped ones were formed with well developed cushions bearing archegonia. The cultures grown from spores contained a large number of small male gametophytes at first; the bisexual plants developed a little later. Therefore the regenerated prothalli were following the normal course of development for this fern. The antheridia were normal and the sperms were motile. There were 32 or 64 sperms in each antheridium as in the normal plants.

Sporophytes developed very soon after the first archegonia appeared on the regenerated prothalli. Experiments to prevent fertilization were also made with this fern. Twelve gametophytes with mature sex organs were kept on agar for two months. Sporophytes did develop on three of these prothalli because antheridia were so numerous; and the prothalli seemed to

grow in a horizontal position instead of standing somewhat on edge as in many ferns. Therefore fertilization could take place very easily if a drop of water fell from the lid of the Petri dish. The remaining nine plants never produced sporophytes. The number of plants used in this experiment to prevent fertilization was small due to lack of material. Observations showed that the sporophytes formed on the regenerated prothalli of *Woodwardia* were not apogamous, but that they grew from the archegonia.



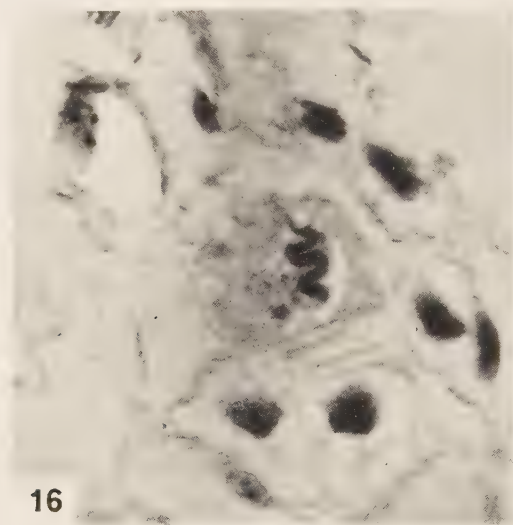
TEXT FIGS. 13-15. *Woodwardia virginica*. Three stages in the development of a filamentous prothallus from a single cell on the margin of a leaf.

Conclusive evidence of fertilization in the $2x$ prothalli of *Woodwardia* was obtained. Some of the prothalli bearing mature sex organs, which had been kept on agar for two months without the formation of sporophytes, were placed in a small glass capsule and covered with nutrient solution for about three hours. At the end of that time they were fixed in formol-acetic-alcohol, embedded in paraffin, and cut. A section was obtained showing sperms in the open neck of an archegonium and a single spirally coiled sperm within the nucleus of the egg (text fig. 16).

Root Regeneration

A number of roots regenerated and produced the same kinds of structures as the leaves. The green roots were cut off and cultivated for about ten weeks before the filamentous prothalli appeared. A little chlorophyll was present in the tips of the roots before they were cut off; but after they were detached, the tips became much greener and chlorophyll was developed for some distance back from the tip. This was in contrast to the roots of

Woodsia obtusa which were green only near the tips. The filamentous prothalli grew from cells on the surface or very near the surface of the root. The $2x$ gametophytes produced only antheridia in the filamentous stage, but later heart-shaped prothalli with archegonia grew from the filamentous ones. Single regenerating roots were isolated and grown on sand and solu-



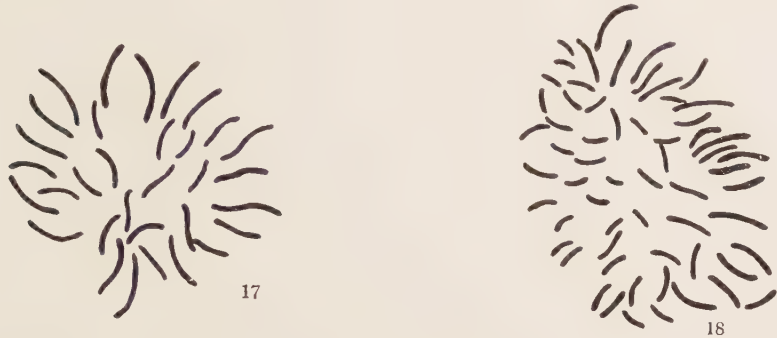
TEXT FIG. 16. *Woodwardia virginica*. Fertilization in a diploid prothallus.

tion until sporophytes developed. These sporophytes were similar to the ones produced on the prothalli regenerated from leaves. Regenerated tissue from the roots of *Woodwardia* was always in the form of prothalli bearing sex organs; while the regenerated tissue formed on the roots of *Woodsia* was either filamentous prothalli without sex organs or sporophytic buds. In each species in which root regeneration occurred, the results were exactly like the regeneration from the leaves of that species.

Chromosome Numbers in Woodwardia

The chromosome numbers show also that fertilization took place before the development of the embryos. Counts were made in normal prothalli, regenerated prothalli, normal sporophytes, and sporophytes developed from regenerated gametophytes, with the results given in table 4. The number of chromosomes is large and therefore cannot be determined exactly from somatic tissue, but reduction stages were not available. In each tissue in which the chromosomes were counted, a number of careful counts were made from different cells. Mitotic figures were easier to obtain in the gametophytes of *Woodwardia* than in *Aspidium* because so many antheridia

were produced on small prothalli. The most accurate counts were made in the first division of the central cell which gives rise to the sperms, because in later divisions the chromosomes of two cells might be confused. The chromosomes of the x gametophyte and of the $2x$ gametophyte are shown in text figures 17 and 18.



TEXT FIG. 17. *Woodwardia virginica*. Chromosomes at metaphase from cell of haploid gametophyte.

TEXT FIG. 18. *Woodwardia virginica*. Chromosomes at metaphase from cell of diploid gametophyte.

TABLE 4. *Chromosome Numbers in Woodwardia virginica*

Normal prothalli.....	32 $\pm$
Regenerated prothalli.....	57- 64
Normal sporophytes.....	59- 65
Sporophytes on regenerated prothalli.....	123-130

Comparison of the Regenerated Plants with Normal Plants

The leaves of the tetraploid plants were much more irregular in outline and often showed foldings and thickenings, as well as lobes which were peculiar in shape and not fully developed. Text figures 19 and 20 show such differences between leaves of young tetraploid and diploid plants.

The results of the microscopic study of the sizes of the cells are shown in table 5. The areas were obtained in the manner described for *Aspidium*.



TEXT FIG. 19. *Woodwardia virginica*. Leaf of young tetraploid sporophyte.

TEXT FIG. 20. *Woodwardia virginica*. Leaf of young diploid sporophyte.

That is, the areas of the prothallial cells are the products of the two dimensions. The areas of the epidermal cells were determined by measuring with a planimeter. Ten cells from the tetraploid plant and the same number from the diploid plant were measured from camera lucida drawings. From these measurements the actual areas as shown in table 5 were calculated. Cells were chosen whose dimensions were consistent with the dimensions shown in the table. Text figures 21 and 22 show the shape of lower epidermal cells of the diploid and tetraploid plants.

TABLE 5. *Cells of Woodwardia virginica*

Prothallus	No. Cells Measured	Lengths in μ	Difference	% Increase
x	300	114.07 ± 1.15		
$2x$	300	135.51 ± 1.47	21.44 ± 1.86	19
		Widths in μ		
x	300	$55.79 \pm .42$		
$2x$	300	$72.44 \pm .54$	$16.65 \pm .68$	30
		Areas in sq. μ		
x		6400		
$2x$		9800		53
Lower Epidermal Cells of Primary Leaves				
Sporophyte	No. Cells Measured	Lengths in μ	Difference	
$2x$	300	$117.43 \pm .97$		
$4x$	300	175.27 ± 1.70	57.84 ± 1.96	49
		Widths in μ		
$2x$	300	$65.45 \pm .45$		
$4x$	300	$85.05 \pm .76$	$19.60 \pm .89$	30
		Areas in sq. μ		
$2x$		3646		
$4x$		6252		71

A comparison of the results of the measurements of the prothallial cells of *Aspidium* and *Woodwardia* showed that there was a greater increase in area in *Woodwardia*. As in *Aspidium* the length of the $2x$ cells did not show as great a change as the width. In neither *Aspidium* nor *Woodwardia* were the areas of the $2x$ cells two or three times the areas of normal cells, a condition reported by Heilbronn (11) for *Polypodium aureum*. The area of the lower epidermal cells of *Woodwardia* was enlarged 71 percent as compared with 75 percent in *Aspidium* and 120 percent in *Polypodium*.

A few leaves of the tetraploid sporophytes regenerated to form gametophytes which probably have the $4x$ chromosome number. Both archegonia and antheridia have been produced on these prothalli.

TEXT FIG. 21. *Woodwardia virginica*. Lower epidermal cells of diploid sporophyte.TEXT FIG. 22. *Woodwardia virginica*. Lower epidermal cells of tetraploid sporophyte.

Attempts to Produce Triploid Sporophytes

In *Woodwardia* the production of small male gametophytes and large bisexual gametophytes with few antheridia made possible an experiment to produce triploid sporophytes. The regions bearing antheridia were cut off some of the large $2x$ prothalli which had been growing on agar for some time. These prothalli, each with many mature archegonia, were put in solution in a small glass capsule together with male gametophytes having the x chromosome number. After a few hours the large $2x$ prothalli were removed and further cultivated. At the end of several weeks each of the prothalli had developed at least one sporophyte. Theoretically the resulting sporophytes should have the $3x$ chromosome number. Due to the manner in which the sporophytes were obtained, it is impossible to be sure that they are triploids until chromosome counts can be made. The possibility of an antheridium on the $2x$ prothalli being overlooked makes the results uncertain, although a careful search was made for antheridia before the $2x$ prothalli were put in the capsule. The sporophytes are quite young and no measurements have been made on the cells of the leaves. The method described here for the production of triploid sporophytes can be applied to any fern which produces prothalli of this type; that is, prothalli bearing antheridia in regions where they can be easily found and removed. It is believed that by further experiments along this line triploid sporophytes can be developed in several species.

Summary of Results of Experiments with Woodwardia virginica

Regenerated prothalli of the typical heart-shaped form were produced on young leaves and young roots of diploid sporophytes. The cells of the

regenerated prothalli were larger than those of normal prothalli and the chromosome number was double that of prothalli grown from spores. Sections of $2x$ prothalli showed the sperm within the egg nucleus. Sporophytes developed from the sex organs of the $2x$ gametophytes. The leaves of the resulting sporophytes differed in appearance and were composed of larger cells than those of normal plants. The chromosome number was the $4x$ number. The leaves of the $4x$ plants also regenerated to produce prothalli bearing archegonia and antheridia.

DISCUSSION

From the large number of Filicales and Bryophytes which have responded to experiments on induced apospory, it is evident that it is not a phenomenon peculiar to a few species. Previous to the present work regeneration had been induced in the leaves of twelve species of ferns belonging to four different families. By the experiments described in this report regeneration was obtained in eleven species, ten of which are here reported for the first time. Regeneration in *Osmunda regalis* was also obtained by Goebel (9).

Species Regenerated

The following species showed regeneration:

Pteris cretica var. *albo-lineata* Hook.
Woodwardia virginica (L.) Sm.
Asplenium platyneuron (L.) Oakes
Athyrium filix femina var.
Scolopendrium vulgare Sm.
Polystichum acrostichoides (Michx.) Schott
Aspidium marginale (L.) Sw.
Cystopteris fragilis (L.) Bernh.
Woodsia obtusa (Spreng.) Torr.
Dicksonia punctilobula (Michx.) Gray
Osmunda regalis L.

Regenerated structures were not obtained from the following:

Pteris aquilina L.
Camptosorus rhizophyllus (L.) Link.
Aspidium spinulosum var. *intermedium* (Muhl.) D. C. Eaton
Onoclea sensibilis L.
Osmunda Claytoniana L.

The primary and secondary leaves of the embryo and in some species the third leaves and the primary roots were capable of growth. Only a few attempts were made to regenerate prothalli from older leaves and they were unsuccessful. However, Heilbronn (11) has reported regeneration from mature leaves of *Polypodium aureum*.

Growth from the leaves was from epidermal cells on either the margin

or the surface and from the petiole. The prothalli produced on the roots probably came from superficial cells also, but on roots the origin is more difficult to determine. The sporophytic buds on the roots of *Woodsia* grew from cells beneath the root cap.

Factors Influencing Regeneration

Although it was found to be very easy to induce regeneration in many ferns, very little was learned about the cause or conditions necessary for regeneration to take place. Since it is known that external conditions influence to a marked degree the growth and development of prothalli arising from spores, it is to be expected that the environment plays an important role in the formation of prothalli from detached leaves. Two different nutrient solutions, Shive's and Knopf's, as well as different concentrations of these solutions, were used for some ferns. Leaves were cut not only at all seasons of the year but for *Woodsia obtusa* every month of the year. Experiments were made in three different laboratories: at the University of Michigan, at Hunter College, and at the Biological Laboratory at Cold Spring Harbor, where there were some variations both in temperature and in the amount of light. Not all of the ferns were tried in all three laboratories but most of them were grown and regenerated in at least two places.

Two collections of spores from plants growing in different localities were made for *Aspidium marginale* and *Asplenium platyneuron*. It was not found that variations in any of the environmental factors which have been mentioned had any definite influence on regeneration. *Woodsia* was tried on different solutions, different concentrations of the solutions, different times of the year, and in different laboratories with the same results. Filamentous prothalli and sporophytic buds were formed but never heart-shaped prothalli. *Aspidium marginale*, whether from spores collected at the Botanical Gardens of the University of Michigan or from Cold Spring Harbor, formed heart-shaped prothalli at all seasons and in both the laboratories where it was tried.

Therefore regeneration is not peculiar to particular plants of a species and it can take place on different concentrations of different nutrient solutions. However, it is necessary to cut the leaves before they are so old that they are beginning to lose their bright green color. Cultures contaminated with algae did not usually regenerate or if growth did begin, the prothalli lived only a short time. Therefore young sporophytes which had been grown in pure culture gave the best results, and cultures on solution could be kept uncontaminated more easily than those on agar or sphagnum.

Structures Produced by Regenerating Leaves

The structures produced by the regenerating leaves were of three distinct types, although some species produced only two types. A few

sporophytes were formed by budding on leaves of *Aspidium marginale* and *Asplenium platyneuron*; and the buds were very common on the leaves and roots of *Woodsia obtusa*. The sporophytes produced by these buds resembled normal plants and the chromosome number was in all probability the diploid number.

Filamentous prothalli were produced as one of the first results of growth in the regenerating leaves in nearly all the species studied. In the early stages they were comparable to the filaments produced by germinating spores but they persisted for a longer time. They bore rhizoids and occasionally antheridia. The filamentous stage was persistent in *Woodsia* and the 4x prothalli of *Aspidium marginale* remained filamentous for nearly six months instead of becoming heart shaped in a few weeks as the 2x prothalli did.

Heart-shaped prothalli developed from the filamentous prothalli of *Aspidium marginale*, *Cystopteris fragilis*, *Woodwardia virginica*, *Osmunda regalis*, *Athyrium filix-femina* var., *Pteris cretica* var. *albo-lineata*, *Asplenium platyneuron*, and *Polystichum acrostichoides*. *Pteris* produced no sex organs and *Asplenium* and *Polystichum* only antheridia. All the others developed both kinds of sex organs.

Polyploidy in the Gametophyte Generation

In two species, *Aspidium marginale* and *Woodwardia virginica*, gametophytes with the 2x chromosome numbers were produced. The evidence for the diploid character of these gametophytes depends on chromosome counts made in the prothalli grown from spores and in the regenerated prothalli. Since the regenerated gametophytes in all the species were produced in a similar manner, that is, by regeneration from diploid leaves, it is believed that diploid gametophytes were obtained in eleven species of ferns. Similarly, gametophytes produced by regeneration from tetraploid sporophytes are believed to have the 4x chromosome number. Such gametophytes were produced in two species, *Aspidium marginale* and *Woodwardia virginica*. Fern gametophytes with the 4x chromosome number have not previously been produced.

The regenerated heart-shaped prothalli resembled the normal ones except that they were composed of larger cells in all the species in which cell measurements were taken. In every species the width of the cells showed a greater increase than the length, a fact also observed for *Polypodium aureum* by Heilbronn (11). Heilbronn states that the area of the normal prothallial cells was to the area of the regenerated as 1 : 2 and often as 1 : 3 or 1 : 4. In no species studied by the writer was so great a difference found. The area of the cells of the regenerated prothalli of *Aspidium* showed an increase of 34 percent; those of *Woodwardia*, 53 percent. A comparison of these results with the tables of cell sizes for regenerated and normal gametophytes of mosses given by Wettstein (24)

showed that for some species of mosses the increase was no greater than for the species of ferns reported here.

Polyploidy in the Sporophyte Generation

It is known that tetraploid plants were produced in two species, *Aspidium marginale* and *Woodwardia virginica*. Chromosome counts were made in the normal plants and in the plants formed on regenerated prothalli of both species and fertilization was observed in the $2x$ gametophytes of *Woodwardia*. Therefore there can be no doubt but that the sporophytes have the $4x$ chromosome number.

The tetraploid plants of *Aspidium* and *Woodwardia* differed from the diploid in the same manner. The irregularities in the leaves were more pronounced in *Woodwardia*. The cells of the primary leaves were much larger in both species. The area of the lower epidermal cells of *Aspidium* was 75 percent greater, and those of *Woodwardia* 71 percent greater, than the corresponding cells of the normal plants. Heilbronn (11) found a 120 percent increase for *Polypodium aureum*. No other fern sporophytes with the $4x$ chromosome number have been produced experimentally. The difference in size in the epidermal cells of *Aspidium* was most pronounced in the length, while in *Woodwardia* the length and width were almost equally enlarged, giving cells of approximately the same shape in the tetraploid as in the diploid.

The measurements made on normal mature plants of *Aspidium* showed no significant difference in size when compared with the measurements made on the primary leaves of the same species. Therefore the size of the cells in the primary leaf indicates the size in the leaves of the mature plants and it is to be expected that the mature tetraploid plants will be composed of cells which are larger than those of the diploid plants.

Several years are required to produce mature sporophytes. Therefore all tetraploid plants are being carefully cultivated in order to bring them to the fruiting stage. The events occurring at meiosis will prove interesting and a study will be made of spore formation when material is available.

Apogamy and Apospory

A discussion of apogamy and apospory as they occur in nature has not been attempted in this paper. Apospory induced by removing and cultivating leaves of young sporophytes is the result of unusual cultural conditions and at present offers no explanation for apospory as it occurs spontaneously. The production of sporangia, epidermal cells, and stomata on regenerated prothalli occurred only on a small percentage of the aposporous gametophytes.

Lang (12, 14) has reported the appearance of sporangia on prothalli of two species of ferns which were apogamous if fertilization was prevented until the gametophytes became very large. The chromosome numbers

have not been determined in these ferns. The situation in *Aspidium*, which also produced sporangia on the prothalli in these cultures, differs in that the prothalli were not old and fertilization had not been prevented. The prothalli studied by Lang were grown from spores, while those of *Aspidium* were produced by regeneration, and had the diploid chromosome number. A sporophyte was produced from an archegonium on the same prothallus which bore sporangia.

Induced apospory ordinarily does not change the sequence of events in the life cycle. This was found to be true for *Polypodium aureum* by Heilbronn (11) and by Wettstein for the mosses (24). Aposporous prothalli of *Aspidium* and *Woodwardia* which were derived from sexually produced sporophytes reproduced sexually; and the aposporous prothalli with the $4x$ chromosome number were not apogamous. On the other hand, *Pteris cretica* var. *albo-lineata* was always apogamous and the regenerated prothallus from that species was apogamous.

SUMMARY

1. Apospory was induced in eleven species of ferns.
2. The aposporous gametophytes of five species were heart-shaped, and bore both archegonia and antheridia. The cells of the aposporous gametophytes were larger than the cells of gametophytes grown from spores.
3. Some ferns produced sporophytic buds and filamentous prothalli on the detached leaves. The sporophytes originating from the buds resembled normal plants.
4. The aposporous prothalli of *Aspidium marginale* and *Woodwardia virginica* produced sporophytes from the sex organs. Fertilization was observed in *Woodwardia*. Chromosome counts showed that these plants were tetraploids.
5. The leaves of the tetraploid sporophytes were abnormal in appearance and were composed of larger cells than the leaves of the diploid sporophytes of the same species.
6. Apogamy does not necessarily follow induced apospory.
7. *Pteris cretica* var. *albo-lineata* was normally apogamous and a regenerated prothallus of this fern was also apogamous.
8. Some of the regenerated prothalli of *Aspidium marginale* bore sporangia and some contained stomata, epidermal cells, and tracheids together with prothallial cells.
9. Sporophytes of *Woodwardia virginica* which probably have the triploid chromosome number were produced by crossing $2x$ plants with x plants.
10. Gametophytes which were believed to have the $4x$ chromosome number were produced by regeneration from the leaves of *Aspidium* and *Woodwardia*. The $4x$ prothalli produced both archegonia and antheridia.

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HUNTER COLLEGE,
NEW YORK CITY

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